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RN-1, a Potent and Selective LSD1 Inhibitor, Increases γ -globin Expression, F-retics, and F-cells in a Sick Cell Disease Mouse Model

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Abstract

Increased levels of fetal hemoglobin are associated with decreased symptoms and increased life span in patients with sickle cell disease (SCD). Hydroxyurea, the only drug currently approved for SCD, is not effective in a large fraction of patients and therefore new agents are urgently needed. Recently it was shown that Lysine Demethylase-1 (LSD1), an enzyme that removes monomethyl and dimethyl residues from the lysine 4 residue of histone H3, is a repressor of γ -globin gene expression. In this report we have compared the ability of tranylcypromine (TCP) and a more potent TCP derivative, RN-1, to increase γ -globin expression in cultured baboon erythroid progenitor cells and in the SCD mouse model. Our results show that the ability of RN-1 to induce F-cells and γ -globin mRNA in SCD mice was similar to decitabine, the most powerful HbF-inducing drug known, and greater than either TCP or hydroxyurea. We conclude that RN-1 and

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Authorship Contribution

Angela Rivers, M.D., Ph.D.: designed research, performed research, contributed vital analyzed data, and wrote the paper.

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Maria Armila Ruiz, B.S.: performed research and contributed vital analyzed data.

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Joseph DeSimone, Ph.D.: designed research and wrote the paper.

Donald Lavelle, Ph.D.: designed research, performed research, contributed vital analyzed data, and wrote the paper.

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other LSD1 inhibitors may be a promising new γ -globin inducing agent for the treatment of sickle cell disease that warrant further studies in other pre-clinical models such as non-human primates.

Introduction

Together β -thalassemia and sickle cell disease (SCD) are the most common monogenic diseases.^{1,2} Both disorders can be ameliorated by increased levels of fetal hemoglobin (HbF).^{3–5} In β -thalassemia, where β -globin expression is highly reduced or absent, γ -globin production can restore normal globin chain assembly into functional hemoglobin.⁵ In SCD, the substitution of glutamic acid for valine at the sixth amino acid of the β -globin protein leads to the formation of abnormal hemoglobin S (HbS).⁶ Following deoxygenation in red blood cells (RBCs), HbS forms polymers causing the RBCs to become deformed and adherent leading to vaso-occlusive events resulting in splenic infarction, kidney failure, stroke, painful crises, and chronic anemia. *In vitro* studies have shown that the HbF ($\alpha_2\gamma_2$) tetramer and the ($\alpha_2\beta^S\gamma$) tetramer inhibit HbS polymerization.^{7,8} Induction of fetal hemoglobin is an important therapeutic strategy in both β -thalassemia and sickle cell disease.^{9,10} Increased levels of HbF are associated with decreased symptoms and increased life expectancy in patients with SCD.⁴

The ribonucleotide reductase inhibitor hydroxyurea (HU) is the only drug approved by the US Food and Drug Administration (FDA) to treat SCD. Patients treated with HU have reduced crisis and longer life expectancy,¹¹ however the drug is not effective in many patients¹² and therefore there is a great need for new and improved HbF-inducing drugs. Recent insights into epigenetics have led to the discovery of a new class of histone modifying enzymes that demethylate histones and modulate gene expression. Lysine Specific Demethylase-1 (LSD1), the first histone demethylase identified, can demethylate the lysine 4 (H3K4) and lysine 9 (H3K9) residues of histone H3. Demethylation of lysine 4 represses gene expression during hematopoietic differentiation.^{13–15} LSD1 and DNA methyltransferase1 (DNMT1) are components of both the direct repeat erythroid definitive (DRED) multiprotein complex¹⁶ and the LSD1/CoRest complex¹⁷ that have been shown to repress γ -globin expression.^{18–21} Inhibition of LSD1 activity using the pharmacological LSD1 inhibitor tranylcypromine (TCP) was recently shown to increase γ -globin expression in human erythroid progenitors and also in a transgenic mouse model that contains human β -globin locus in a yeast artificial chromosome (β YAC).¹⁶

Although LSD1 inhibitors are attractive candidate drugs for HbF induction, the usefulness of TCP will most likely be limited in clinical trials by side effects induced as a potent monoamine oxidase (MAO) inhibitor. MAO inhibitors have hypotensive effects, and up to 50 percent of patients experience dizziness. RN-1 is a recently described TCP derivative that is a more potent inhibitor of LSD1 than TCP.²² In three different *in vitro* assays of LSD1 activity, the IC₅₀ of RN-1 ranged between 2 to 10nM while that of TCP was >100 μ M. Because the IC₅₀ of RN-1 for MAO-A was 0.5 μ M and for MAO-B was 2.8 μ M, RN-1 is a highly selective and potent inhibitor of LSD1 compared to TCP with great potential as an *in vivo* agent. In this report we show that RN-1 increases γ -globin expression in cultured nonhuman primate erythroid progenitors and in sickle cell mice and compare its effects in

sickle cell mice with other known HbF-inducing drugs. Our results show that RN-1 is a potent activator of γ -globin expression with activity comparable to decitabine, the most powerful in vivo pharmacological HbF-inducer known, and is therefore an excellent candidate for further testing in nonhuman primates.

Materials and Methods

Cell lines and drugs

The U937 AML cell line was obtained from the laboratory of Dr. Zhijian Qian and was grown in RPMI medium containing 10% fetal bovine serum. Based on a recent report that differentiation of AML cell lines including U937 is induced by a combination of TCP and all-trans-retinoic acid (ATRA), the U937 cell line was used to compare the ability of other commercially available pharmacological LSD1 inhibitors to induce differentiation in combination with ATRA.²³ LSD1 Inhibitor I (489476-a bisguanidine polyamine analog), LSD1 inhibitor II (489477-S2101), LSD1 Inhibitor III (489478-CB1007), and LSD1 inhibitor IV (489479-RN-1) were obtained from EMD Millipore (Darmstadt, Germany) and dissolved as stock solutions in DMSO. Tranylcypromine (Sigma, St. Louis, Mo.) was dissolved directly in RPMI medium. Initially 1×10^{-6} M doses of drugs were added (1×10^{-6} M) alone and in combination with all-trans-retinoic acid (1×10^{-7} M; Sigma, St. Louis, Mo.) and the effect on differentiation was analyzed by measuring CD11b expression by flow cytometry using phycoerythrin-conjugated anti-CD11b antibody (#555388 BD Bioscience, Brea, Ca.). In subsequent experiments RN-1 and TCP were compared at doses of 10, 2.5, 0.625, 0.156, 0.039 and 0.009 μ M.

Isolation and culture of baboon bone marrow CD34+ cells

Baboons were housed at the University of Illinois at Chicago Biologic Resources Laboratory (UIC-BRL) under conditions that meet the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) standards. Bone marrow aspirations were performed from the hips of animals under ketamine/xylazine anesthesia (10 mg/kg; 1 mg/kg). Prior to bone marrow sampling, buprenex (0.01 mg/kg IM) was given and later in the afternoon a second dose of buprenex (0.01 mg/kg IM) was administered to alleviate potential pain and suffering.

Mononuclear cells were enriched from baboon BM aspirates (30–40 ml) by sedimentation in Percoll gradients. Purification of CD34+ cells was performed by immunomagnetic column chromatography using the anti-CD34 12.8 monoclonal antibody (a gift of Dr. Robert Andrews) as the primary reagent and immunomagnetic rat anti-mouse IgM microbeads (Miltenyi Biotech) as the secondary reagent. Isolated CD34+ cells were grown in T25 flasks in co-culture with the AFT024 stromal cell line in Iscove's media containing 30% fetal bovine serum (FBS), 2 U/ml erythropoietin (EPO), 200ng/ml stem cell factor (SCF), and 1 μ M dexamethasone as previously described²⁴. Varying doses of RN-1 or TCP were added on day 7 and cells harvested for analysis of globin on day 14. Lysates were prepared by suspension of cells in 5mM EDTA and lysed by five cycles of freeze-thaw in dry ice-methanol baths. Effects on differentiation were assessed by flow cytometry by analysis of expression of CD36 and a baboon red cell-specific antigen (bRBC) present on mature red

blood cells and non-colony forming erythroid precursors. Cells were stained for flow cytometry analysis using FITC anti-CD36 (Beckman Coulter, Brea, Ca., IM0766U, clone FA6.152) and APC- Baboon RBC antibody (BD Bioscience, San Jose, Ca., #551299).²⁴

Sickle cell mice

SCD mice were bought from Jackson Laboratory (B6;129-*Hba*^{tm1(HBA)Tow}/*Hbb*^{tm2(HBG1,HBB*)TowJ}) and were bred at the University of Illinois Chicago according to institutional guidelines. In the initial group treatment started at 8 weeks of age. Treatment groups consisted of vehicle (DMSO; n=10), TCP (6 mg/kg; n=3), HU (100 mg/kg; n=3), decitabine (DAC) (0.25 mg/kg; n=7), and RN-1 (2.5 mg/kg; n = 5, 5 mg/kg; n= 10, and 20 mg/kg; n=4). Initial sample size calculation was done using statistical solutions software with an α -value of 0.05 and power of 0.8. For mRNA, to detect a 50% increase 3 mice were needed. It is difficult to generate 18 match littermates, and therefore not all treatment groups were compared simultaneously. However, experiments testing the effect of individual drugs in a group of mice were always performed alongside a DMSO-treated control group.

Drugs were administered by intraperitoneal injection daily for 10 days. Blood samples drawn on d5 and d11 were collected by retro-orbital bleeding in isoflurane-anesthetized mice in vacutainer EDTA tubes (BD Biosciences, San Jose, Ca.). Complete blood count analyzed with the CBC-Diff by the BRL staff using a Siemens-Abvia 120 automated hematology analyzer.

To monitor the effects of a more prolonged period of drug treatment a second set of experiments was done in 16 week old mice. Mice were treated with either DMSO (control; n=3) or RN-1 (5mg/kg; n=3) by intraperitoneal injection daily for 4 weeks. Blood samples drawn on day 28. The effect on red blood cell morphology was analyzed by determining the percentage of sickle cells on a peripheral smear. Five hundred RBC's were counted from randomly selected fields. Abnormal RBC was defined as elongated or tapering of the opposite ends that culminated in a point. Spleens were weighed at the time of sacrifice, fixed in formalin and staining was performed by the Research Histology and Tissue imaging core of UIC.

F-cells and F-retics

F-cells and F-retics were analyzed by flow cytometry using the Cytomics FC500 (Beckman Coulter, Brea, Ca.) system after staining with thiazole orange and PE-conjugated anti-HbF (BD Bioscience, San Jose, Ca.).

RNA analysis

RNA was purified from mouse peripheral blood using a Mouse RiboPure Blood RNA Isolation kit (Life Technologies, Waltham, Ma.). RNA samples were treated with the RNase-free DNase (Ambion Life Technologies, Grand Island, NY. and cDNA was synthesized using the RevertAid first strand cDNA synthesis kit (Thermo Scientific, Waltham, Ma.) according to the manufacturer's instructions. Levels of human α -globin (Hs00361191_g1), γ -globin(Hs00361131_g1), and β -globin (Hs00747223_g1) mRNA were measured by real time PCR using the indicated Taqman primer-probe combinations (Life Technologies,

Waltham, Ma.). Expression of mouse Ey and Bh1 mRNA was measured using the Taqman primer-probe sets Mm00433936_g1 and Mm00433932_g1, respectively.

Quantitative analysis of murine terminal erythroid differentiation *in vivo*

Quantitative analysis of murine terminal erythroid differentiation was performed according to the method of Liu et al²⁵. To prepare a spleen single cell suspension, a whole spleen was passed through a cell strainer. The cells were transferred to a 15-mL tube and centrifuged at 300g for 5 minutes. After centrifugation, the cell pellet was suspended in PBS/2% BSA buffer. The cells were then incubated with the following antibodies for 45 minutes FITC-conjugated anti-Ter119, APC-conjugated anti-CD44, APC-Cy 7-conjugated anti-CD45, APC-Cy 7-conjugated anti-CD11b, APC-Cy 7-conjugated anti-GR1, and 7AAD. Antibodies were purchased from BD Bioscience. Cells were analyzed by flow cytometry in a Beckman Coulter FC-500 with 2 lasers.

Statistics

Student's *t*-test was used for all the statistical analyses. Error bars represent standard deviation.

Results

RN-1 is potent inducer of cell differentiation

To identify LSD1 inhibitors with increased potency compared to TCP, an *in vitro* screening assay for LSD1 activity was performed based on the recent report that TCP in combination with low-dose ATRA induced differentiation in the AML cell line U937.²³ The U937 cell line was treated with four commercially available LSD1 inhibitors (1×10^{-6} M) alone and in combination with low-dose ATRA (1×10^{-7} M). Four days following addition of drugs, effects on differentiation were assessed by flow cytometric analysis of CD11b expression, a marker of the mature myeloid lineage.²³ Increased CD11b median fluorescence was observed in cells treated with ATRA in combination with either TCP or RN-1. Initial experiments showed that both TCP and RN-induced differentiation in combination with ATRA, while the other inhibitors tested had no effect (Supplemental Figure 1A). Additional dose-response experiments were performed to compare the activities of RN-1 and TCP and showed that RN-1, in combination with ATRA, induced a similar increase in CD11b median fluorescence at a dose of drug 64 fold lower than TCP (0.156 μ M RN-1 vs 10 μ M TCP), consistent with its reported increased potency in various *in vitro* assays (Supplemental Figure 1B).

Effect of RN-1 on γ -globin expression in cultured erythroid progenitors

Experiments were conducted to compare the effects of TCP and RN-1 on γ -globin expression in cultured baboon erythroid progenitors. TCP doses were chosen based on previous studies by Shi et al¹⁶ while reduced doses of RN-1 were tested based on our observation of increased potency of RN-1 compared to TCP in the induction of U937 cell differentiation (Supplemental Figure 1B). Cultures were treated with varying doses of RN-1 or TCP on day 7 and globin chain expression measured on day 14 by HPLC. Dose-response experiments showed that similar levels of γ -globin were induced by RN-1 at a dose of drug

1/23 that of TCP (Figure 1). Cell numbers were reduced in RN-1-treated cultures and the calculated RN-1 IC₅₀ (0.127 μ M), was considerably lower than the IC₅₀ of TCP (20.8mM; Supplemental Figure 2). Thus RN-1 was both a more potent inducer of γ -globin expression than TCP and also was a more potent inhibitor of cell growth in erythroid progenitor cell cultures. Doses of RN-1 that reduced cell growth also affected erythroid differentiation. Flow cytometry showed a reduction in CD36+bRBC⁺ cells and increased numbers of less mature CD36+bRBC⁻ cells. Analysis of Wright's stained cytopsin preparation showed increased numbers of basophilic cells and reduced numbers of terminally differentiated polychromatic and orthochromatic stained cells compared to controls (Supplemental Figure 3).

Effects of RN-1 on Sick cell mouse model

The ability of TCP and RN-1 to induce γ -globin expression in humanized SCD mice containing the human α , γ , and β -globin genes was compared with the known HbF-inducing drugs DAC and hydroxyurea (HU). SCD mice (8 weeks old) were injected for 10 days with varying doses of RN-1 (2.5 mg/kg, 5 mg/kg, or 10 mg/kg), TCP (6 mg/kg), DAC (0.25 mg/kg), or HU (100 mg/kg). F-retics, F-cells and γ -globin mRNA in peripheral blood were analyzed on day 5 and day 11. On d5 F-retics were significantly increased in mice treated with DAC (8.4 $\pm$ 2.8; p <0.0005), HU (6 $\pm$ 1.5; p <0.01), and the 2.5 mg/kg (7.5 $\pm$ 2.4; p <0.001), 5 mg/kg (9.2 $\pm$ 1.8; p <0.005), and 10mg/kg doses of RN-1 (13.2 $\pm$ 2.7; p <0.0005) compared to controls (4.1 $\pm$ 0.7). On day 11 F-retics remained elevated in mice treated with DAC (12 $\pm$ 4.3; p <0.002), HU (5.1 $\pm$ 1.1; p <0.03), 2.5 mg/kg RN-1 (5.6 $\pm$ 1.3; p <0.02), and 5 mg/kg RN-1 (10.5 $\pm$ 3.8; p <0.002) compared to controls (3.9 $\pm$ 0.8). Mice treated with 10 mg/kg RN-1 were sacrificed prior to day 11 secondary to weight loss. F-retics of TCP-treated mice were not significantly increased on either day 5 (3.7 $\pm$ 0.2) or d11 (3.9 $\pm$ 0.7). F-retics in mice treated with 5mg/kg RN-1 (9.2 $\pm$ 1.8) were higher than HU on both day 5 and day 11 (p <0.002 and p <0.009), respectively (Figure 2A). The level of F-cells on day 11 was increased in mice treated with DAC (11.8 $\pm$ 2.6; p <0.005), HU (6.2 $\pm$ 1.08; p <0.03) and RN-1 at both the 2.5 mg/kg (7.76 $\pm$ 1.13; p <0.005) and 5 mg/kg doses (12.5 $\pm$ 1.85; p <0.005) compared to control mice (4.8 $\pm$ 0.6; Figure 2B). No increase in F cell levels was observed in mice treated with TCP (4.9 $\pm$ 0.95). Increased levels of γ -globin mRNA ($\gamma/\gamma+\beta$ fold change) were observed on day 11 in mice treated with DAC (4.24 $\pm$ 1.4; p <0.001), HU (1.8 $\pm$ 0.6; p <0.02) and RN-1 at both the 2.5 mg/kg (1.78 $\pm$ 0.98; p <0.001) and 5 mg/kg doses (4.34 $\pm$ 1.36; p <0.005) compared to controls (Figure 2C). No increase in γ -globin mRNA was observed in mice treated with TCP. On day 11 levels of both F-cells (p <0.001) and γ -globin mRNA (p <0.02) were significantly higher in mice treated with 5mg/kg RN-1 compared to HU-treated mice but were not significantly different than the levels attained in DAC-treated mice. Increased levels of embryonic mouse Bh1 and Ey1 mRNA were observed in mice treated with 2.5 mg/kg and 5mg/kg RN-1 but were not observed in DAC-treated mice (Supplemental Figure 4). RBC's were statistically decreased in only the HU (5.9 $\pm$ 0.1 $10^6/\mu$ l; p <0.01) and the DAC-treated groups (5.6 $\pm$ 0.22 $10^6/\mu$ l; p <0.05) compared to DMSO (9.7 $\pm$ 0.8 $10^6/\mu$ l). Decreased RBC's were also observed in the TCP (7.97 $\pm$ 0.5 $10^6/\mu$ l; p =0.06) and RN-1 5 mg/kg (7.87 $\pm$ 1.9 $10^6/\mu$ l; p <0.08) groups but the differences were not statistically significant. RBC's in the 2.5 mg/kg group (9 $\pm$ 0.38 $10^6/\mu$ l; p =0.21) were not different than control group (Figure 3A). The effect on Hgb followed a similar pattern with decreased Hgb observed only

in the HU and DAC treatment groups (Figure 3D). Absolute retic count was decreased in all treated groups except TCP. Absolute retic counts in mice treated with DAC ($1281 \pm 748 \times 10^3/\mu\text{l}$; $p < 0.001$), HU ($1462 \pm 26 \times 10^3/\mu\text{l}$; $p < 0.001$), TCP ($3988 \pm 25 \times 10^3/\mu\text{l}$; $p = .55$) and the 2.5 mg/kg ($2774 \pm 609 \times 10^3/\mu\text{l}$; $p < 0.01$), 5 mg/kg ($2210 \pm 552 \times 10^3/\mu\text{l}$; $p < 0.005$) doses of RN-1 were significantly reduced, compared to controls ($4257 \pm 551 \times 10^3/\mu\text{l}$). RBC, HGB and absolute retic count were higher in the RN-1 2.5 mg/kg group than in the HU-treatment group (Figure 3B). No change in MCHC was observed in any of the drug-treated mice indicating that overall hemoglobin synthesis was not altered (Figure 3C). WBC, ANC, platelets and ALC were decreased in all treatment groups (Figure 4A-D). The erythroid differentiation pattern in the spleen, evaluated by flow cytometry, was similar to controls in all treated mice groups except HU, where an increase in orthochromatic erythroblasts and a decrease in mature RBCs were observed (Figure 5).

A second experiment was performed to evaluate the effects of more prolonged treatment. Four month old sickle cell mice were injected for 4 weeks with either DMSO (control) or RN-1 (5mg/kg). F-cells were increased in the RN-1-treated mice (7.63 ± 0.68) compared to DMSO controls (3.05 ± 0.26 ; $p < .05$) and F-Retics were also increased 2 fold ($p < 0.05$) in RN-1-treated mice. No significant difference in Hgb, the number of sickle cells in peripheral blood smears, or spleen weight and splenic architecture was observed between the RN-1 treated and control mice (Supplemental Figure 5).

Discussion

Our results in SCD mice identify RN-1 as a more potent inducer of γ -globin expression than either TCP or HU. The activity of RN-1 *in vivo* in the SCD mouse model is comparable to DAC, the most powerful known HbF-inducing drug. There is no clear explanation for the lack of γ -globin mRNA induction by TCP on this Townes SCD model as compared to its positive effect on the β YAC model.¹⁶ It is possible that the lack of effect in our studies is due to the difference in schedule of administration of TCP. However, differences in the chromatin environment of the β YAC transgene, containing the complete human β -globin locus including the human LCR, compared to the human genes that are located within the context of the normal mouse β -globin locus in the SCD knock-in model, could result in a differential sensitivity of the γ -globin gene to LSD1 inhibitors. In transgenic mice the human γ -globin gene is regulated as an embryonic-stage gene²⁰ and this may contribute to the lower levels of γ -globin induction in these mice. However, use of the SCD mouse model can provide key data demonstrating *in vivo* biological activity of RN-1 and other HbF-inducing drugs and is thus an important step in the translation from a cell culture system to further testing in non-human primates.

DAC is currently being evaluated in clinical trials and its long term safety profile is unknown. DAC is incorporated into DNA and forms covalent adducts with DNMT1 that are excised by DNA repair pathways.^{26,27} Because RN-1 is not incorporated into DNA, it may be a more desirable drug for long term use. LSD1 is highly over expressed in many cancers including prostate, undifferentiated malignant neuroblastoma, estrogen-receptor-negative breast, bladder, lung, colorectal and AML.²⁸ Inhibition of LSD1 may be an effective therapy

in these diseases and efforts are underway to develop even more potent and highly selective LSD1 inhibitors that are more likely to be clinically useful.

The activity of RN-1 as an inducer of γ -globin expression is likely the result of its ability to inhibit LSD1 in multiple co-repressor complexes such as DRED and the Bcl11A-interacting NuRD complex. However, LSD1 and DNMT1 also directly interact and covalent modification of DNMT1 by LSD1 increases DNMT1 stability.²⁹ The stability of DNMT1 was decreased and DNA hypomethylation was increased in DNMT1 knockout ES cells. Double knockdown of BCL11a and DNMT1 mice enhanced γ -globin expression up to 90% and was significantly increased over the single knockdowns of BCL11a or DNMT1.³⁰ Thus the mechanism responsible for powerful induction of HbF by the LSD-1 inhibitor RN-1 could be due to its ability to target multiple epigenetic pathways affecting both histone modifications and DNA methylation.

In conclusion these studies demonstrate that more potent and selective LSD-1 inhibitors such as RN-1 may be effective at inducing HbF in vivo. Therefore, further testing of these drugs in the non-human primate model should be performed as a necessary step prior to clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. RN-1 is a more potent inducer of γ -globin synthesis than tranylcypromine (TCP) in cultured baboon erythroid progenitors
2. RN-1 increases F-retics, F-cells, and γ -globin mRNA in a sickle mouse model.
3. The effects of RN-1 in the SCD mouse model are greater than either tranylcypromine (TCP) or hydroxyurea and similar to decitabine, the most powerful *in vivo* HbF-inducing agent known. Further testing of RN-1 in nonhuman primates should be performed.

Figure 1. Effects of TCP and RN-1 on γ -globin chain expression measured by HPLC in cultured baboon erythroid progenitors.

Baboon erythroid progenitors were treated with varying doses of RN-1 and TCP on d7. Globin chain expression ($\gamma/\gamma+\beta$) was measured by HPLC in lysates prepared on d14. The change in γ -globin chain expression ($\Delta\gamma/\gamma+\beta$) was calculated by subtracting the RN-1 treated samples from control samples. Pooled data from three independent experiments are shown.

Figure 2. Effects of HU, DAC, TCP, and RN-1 on F-retics, F-cells, and γ -globin mRNA in the SCD mouse model.

Mice were injected daily IP for ten days with HU (100mg/kg), DAC (0.25mg/kg), TCP (6mg/kg) or RN-1 (2.5, 5.0 and 10.0 mg/kg). The effect on % F-retics (A), % F-Cells (B), and γ -globin mRNA ($\gamma/\gamma+\beta$) (C) were measured on day 5 (lined bars) and day 11 (solid bars). * $p < .05$ relative to DMSO vehicle; ★ $p < 0.05$ relative to HU.

Figure 3. Effects of HU, DAC, TCP, and RN-1 on various RBC parameters.

SCD mice were treated daily for ten days with either HU (100mg/kg), DAC (0.25mg/kg), TCP (6 mg/kg), or RN-1 (2.5, 5.0 or 10.0 mg/kg). The effect on RBC (A), Absolute Retic Count (B), MCHC (C), HGB (D), MCV (E), and MCH (F) are shown. * $p < .05$ relative to DMSO vehicle

Figure 4. Effects of HU, DAC, TCP, and RN-1 on CBC parameters.

Mice were injected daily IP with HU (100mg/kg), DAC (0.25mg/kg), TCP (6mg/kg), or RN-1 (2.5, 5.0, or 10.0 mg/kg). The effects on WBC (A), PLT (B), ANC (C), and ALC (D) are shown. * $p < .05$ relative to DMSO vehicle.

Figure 5. Representative erythropoiesis profiles of spleen from sickle cell mice.

(Top panel) Plot of CD44 versus FSC of the TER119 positive cells with gating the population 1) proerythroblast (Pro), 2) basophilic erythroblasts (Baso), 3) polychromatic erythroblasts (Poly), 4) polychromatic erythroblasts 5) Reticulocyte Count and 6) Red Blood Cells. (Bottom panel) Representative erythropoiesis profiles of spleen in a bar graph format.